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Global DNA methylation of WTC prostate cancer tissues show signature differences compared to non-exposed cases

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Abstract

There is increased incidence of prostate cancer (PC) among World Trade Center (WTC)-exposed responders and community members, with preliminary evidence suggestive of more aggressive disease. While previous research is supportive of differences in DNA methylation and gene expression as a consequence of WTC exposure, as measured in blood of healthy individuals, the epigenetics of WTC PC tissues has yet to be explored. Patients were recruited from the World Trade Center Health Program. Non-WTC PC samples were frequency matched on age, race/ethnicity and Gleason score. Bisulfite-treated DNA was extracted from tumor tissue blocks and used to assess global DNA methylation with the MethylationEPIC BeadChip. Differential and pathway enrichment analyses were conducted. RNA from the same tumor blocks was used for gene expression analysis to further support DNA methylation findings. Methylation data were generated for 28 samples (13 WTC and 15 non-WTC). Statistically significant differences in methylation were observed for 3,586 genes; on average WTC samples were statistically significantly more hypermethylated ($P = 0.04131$). Pathway enrichment analysis revealed hypermethylation in epithelial mesenchymal transition (EMT), hypoxia, mitotic spindle, TNFA signaling via NFKB, WNT signaling, and TGF beta signaling pathways in WTC compared to non-WTC samples. The androgen response, G2M and MYC target pathways were hypomethylated. These results correlated well with RNA gene expression. In conclusion, long-term epigenic changes associated with WTC dust exposure were observed in PC tissues. These occurred in genes of critical pathways, likely increasing prostate tumorigenesis potential. This warrants analysis of larger WTC groups and other cancer types.

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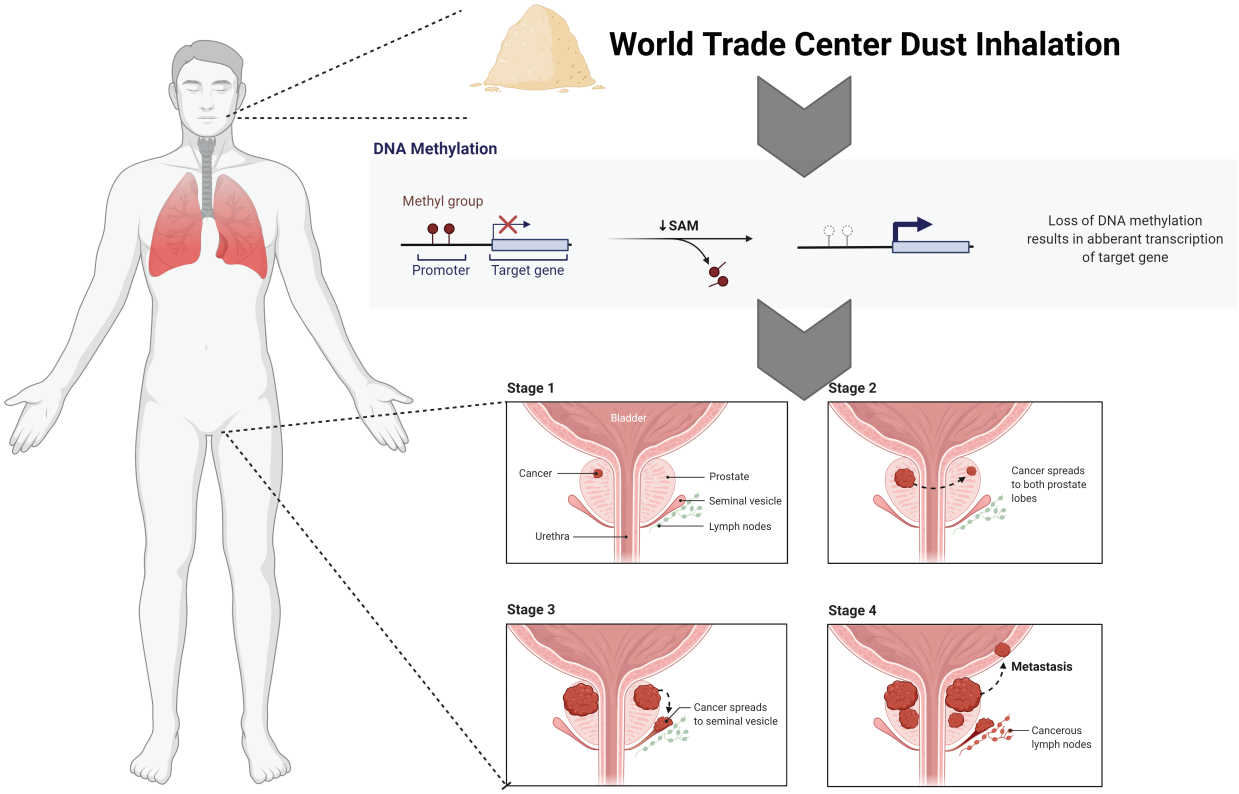
Graphical Abstract

World Trade Center Prostate Cancer Patients

Increased Global Hypermethyation in prostate cancer tissues



Abbreviations: EMT, epithelial mesenchymal transition; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; WTC, World Trade Center

Introduction

New York City residents and first responders to the World Trade Center (WTC) disaster were exposed to a complex mixture of known and suspected human carcinogens. To date, three cohort studies indicate that overall cancer rates of those exposed to WTC dust are 6-14% higher than background rates (1–3). In particular, an increased risk of prostate cancer (PC) was reported in both the WTC Health Program (WTCHP) at Mount Sinai Medical Center and the WTC-exposed firefighter cohort (4). Additionally, prostate cancer is the most common cancer observed among WTC-exposed community members (5). Preliminary evidence suggests that the PC cases of the WTC cohort are on average more aggressive, with responders who had higher levels of WTC dust exposure presenting with more advanced clinical stages (4).

WTC dust contained carcinogens and toxic agents specifically implicated in prostate malignancy, such as silica, cement, glass fibers, heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and polychlorinated dibenzodioxins (dioxins) (6–8); dioxins, PCBs and PAHs have all been shown to be independently associated with increased PC risk (9,10). Asbestos, a naturally occurring mineral previously used in commercial insulation, which is associated with PC development (11), was also present in the dust. Nitrogen dioxide, another compound present in the dust cloud, has been shown to cause oxidative damage to the prostatic epithelium (12,13). However, the causal relationship between acute dust exposure and PC development in WTC responders is complicated to establish, and confounded by the long latency between exposure and disease occurrence. Studies aimed at identifying the specific mechanistic relationships between WTC dust exposure and PC etiology are currently lacking.

Our research team investigated the immunologic and inflammatory response in WTC prostate tumor samples and compared it to non-WTC PC tissues, with results indicating that WTC PCs have a distinct proinflammatory pattern that could be the result of WTC dust exposure (14). We hypothesize that the observed changes in gene expression could be the consequence of epigenetic modifications, including DNA methylation, associated with exposure to WTC dust. Environmental exposures to chemical agents, such as those found at the WTC disaster site, including metals, air pollutants, benzene, and organic pollutants, have all been shown to modify the DNA epigenetic status (15–18). Previous studies using blood from women without cancer diagnosis showed that WTC- exposed and unexposed individuals have, on average, persistent and substantial differences in their methylation profiles (19). Although this study was limited to those healthy and free of cancer, top differentially methylated genes were found to belong to several cancer-related pathways (19). Kuan et al. also presented results suggestive of epigenetic changes associated with WTC dust exposure in peripheral blood, but did not find statistically significant differences in methylated CpG sites after adjustment for multiple testing (20). This may in part be due to the limitation of comparing

the pattern of methylation in WTC dust highly exposed individuals vs. those with low levels of exposure, as quantifying WTC exposure is difficult. This is both because of recall bias as well as lack of knowledge of WTC responders concerning the physical distribution and density of the WTC dust cloud. While DNA methylation is associated with both PC carcinogenesis and aggressiveness (21), no studies have yet investigated the role of DNA methylation in PCs associated with WTC dust exposure.

We performed the first global DNA methylation profile of PC tissues from WTC responders compared to PC tissues from unexposed individuals, in order to assess if DNA methylation signatures differ based on WTC dust exposure. Additionally, we sought to identify differences in methylation that might explain, at the molecular level, PC development in the WTC cohort. To further support the findings from the DNA methylation analysis, we also performed differential gene expression analysis.

Material and Methods

Study participants

Responders who participated (as employees or volunteers) in the rescue, recovery and cleanup efforts at the WTC sites were enrolled at Mount Sinai in the World Trade Center Health Program (WTCHP), which is funded under the James Zadroga 9/11 Health and Compensation Act of 2010, on the basis of eligibility criteria including type of duties, site location and dates and hours worked (22). The medical protocol for the monitoring program includes self-administered physical and mental health questionnaires as well as a physical examination, laboratory tests, spirometry and a chest radiograph (22). Of the 27 000 responders that have had a least one monitoring visit in the WTCHP, 17 781 are male responders who have consented to have their records used for medical research (23).

Sample acquisition and analysis

Patient recruitment and sample retrieval has been previously described; in brief patients were contacted by letter and/or by phone to give written study consent, and formalin-fixed paraffin- embedded (FFPE) tumor tissue samples were requested from the hospitals where they received their PC treatment (22). Non-WTC PC samples were obtained from the Mount Sinai tumor tissue bank. Of the originally included 17 WTC patients and 17 non-WTC patients, frequency matched on age (±5 years), race/ethnicity and Gleason score, methylation data was successfully obtained from 15 PC patients in the non-WTC and 13 PC patients in the WTC group (Table 1). Efforts were made to ensure that tumor cells comprised more than 80% of tumor samples analyzed. All DNA samples underwent processing with the Infinium FFPE QC and DNA Restoration Kit (Illumina, Inc., San Diego, CA) to improve DNA quality prior to hybridization. Bisulfite-treated DNA from cancer tissue blocks was assessed with the MethylationEPIC BeadChip according to manufacturer's recommendations, which yields methylation data for approximately 850,000 CpGs (Illumina, Inc., San Diego, CA). RNA from the same tumor blocks from which DNA was collected was used for RNA-seq analysis. This study was approved by the Mount Sinai Institutional Review Board (IRB # 13-00430).

Preprocessing of methylation data

Methylation data were pre-processed through R package *ChAMP* (Chip Analysis Methylation Pipeline for Illumina HumanMethylation450 and EPIC) (24). One sample in which the fraction of failed CpG was above 0.1 was removed, and probes of detection p-value above 0.01 were filtered out. In total, the methylation profiles included approximately 720 000 probes across 28 samples. Since one gene corresponds to multiple probes and methylation levels, and many probe sites may not contribute to transcription regulation, we selected for each gene the probe whose methylation level most likely correlates with its gene expression in *cis* to represent the gene-level methylation. For this purpose, we used the TCGA PRAD dataset as reference where paired RNA-seq and methylation profiles were available for a large number of samples (n = 497 patients). Specifically, for each gene, we selected the methylation probe that was most anti-correlated with its gene expression in *cis* in TCGA PRAD data set (24–26). After this filtering the gene-level methylation data included 14,042 genes.

Preprocessing of RNA-seq expression data

The software TopHat2(27) was used to map the RNA-seq reads to the genome and to calculate the Reads Per Kilobase

Table 1. Characteristics of participants with samples available for differential methylation analysis

Variable	Non-WTC Prostate Cancer (n = 15)	WTC Prostate Cancer (n = 13)	P-value^
	[mean (SE)]	[mean (SE)]	
Age at diagnosis; years (sd*)	54.5 (1.4)	52.7 (1.5)	0.7833
	n (%)	n (%)	
Race/Ethnicity*			0.6282
White	13 (86.7)	9 (75.0)	
Other	2 (13.3)	3(25.0)	
Gleason Score			0.6860
6	5 (33.3)	3 (23.1)	
7	9 (60.0)	10 (76.9)	
8	1 (6.7)	0 (0.0)	

^*P*-values derived from non-missing values: Fisher's exact test (race/ethnicity, Gleason score); Wilcoxon rank-sum test (age at diagnosis) *1 missing race from WTC group; *sd, standard deviation.

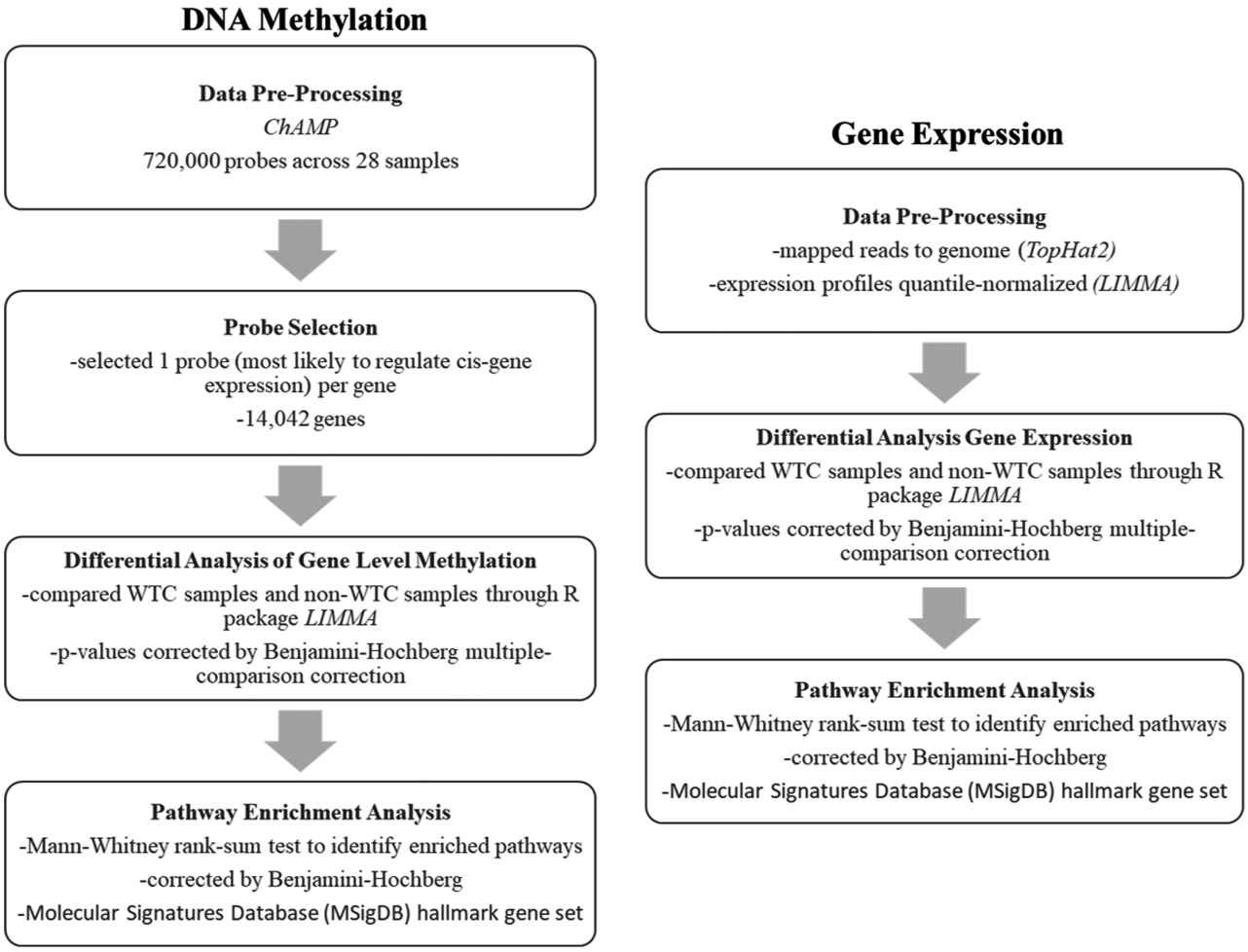


Figure 1. Data analysis pipeline.

of transcript, per Million mapped reads (RPKM) value for each gene, after removing samples of low quality (read count per sample less than 5 million). Expression profiles were further quantile-normalized by R package *LIMMA* (28).

Differential gene expression analysis and pathway enrichment analysis

Differential analysis of gene expression or gene-level methylation was carried out through R package *LIMMA*. For each gene, its expression or methylation level was compared between WTC samples and non-WTC samples to get t-statistics and the *P*-values. The *P*-values of all genes or probes were corrected by Benjamini-Hochberg multiple-comparison correction. Derived gene lists underwent pathway enrichment analysis using the Molecular Signatures Database (MSigDB) hallmark gene set (29). To identify the enriched pathways, the *t*- statistics of genes within a pathway of interest were compared with those outside the pathway through Mann-Whitney rank-sum test. *P*-values from Mann-Whitney rank-sum tests were corrected by Benjamini-Hochberg multiple-comparison correction.

Cell composition adjustment

We performed computational deconvolution of cell components from RNA-seq and Methylation profiles. Frequency of different cell components were inferred from gene expression profiles through CIBERSORT (30). To construct a proper reference profile for prostate cancer samples, we modified the default LM22 gene signature matrix by adding 3 more reference profiles representing prostate cancer cells, endothelial cells, and fibroblast cells, respectively. The expression profiles of the above three cell types were downloaded from GSE16683 (31), GSE24598(32) and The Cancer Cell Line Encyclopedia to enable predictive modeling of anticancer drug sensitivity (33). In addition, cell frequency was also inferred from methylation profiles through R package *MethylCIBERSORT* (34). The default methylation signature matrix was modified by adding the reference profile for prostate cells (constructed from GEO data set GSE68379) (35). This allowed for adjustment of results by immune cell fraction per deconvolution. Our previous work has shown that tumor purity, stromal content, and immune content are highly correlated (36), thus this method represents a means to adjust for tumor purity of the samples (Figure 1).

Results

There were 28 samples (13 WTC and 15 non-WTC) for which methylation data were available (Table 1), and 21 samples (10 WTC and 11 non-WTC) with RNA-seq data, after QC. Among them, 8 WTC and 9 non-WTC had both RNA-seq and methylation profiles available. PC patients with and

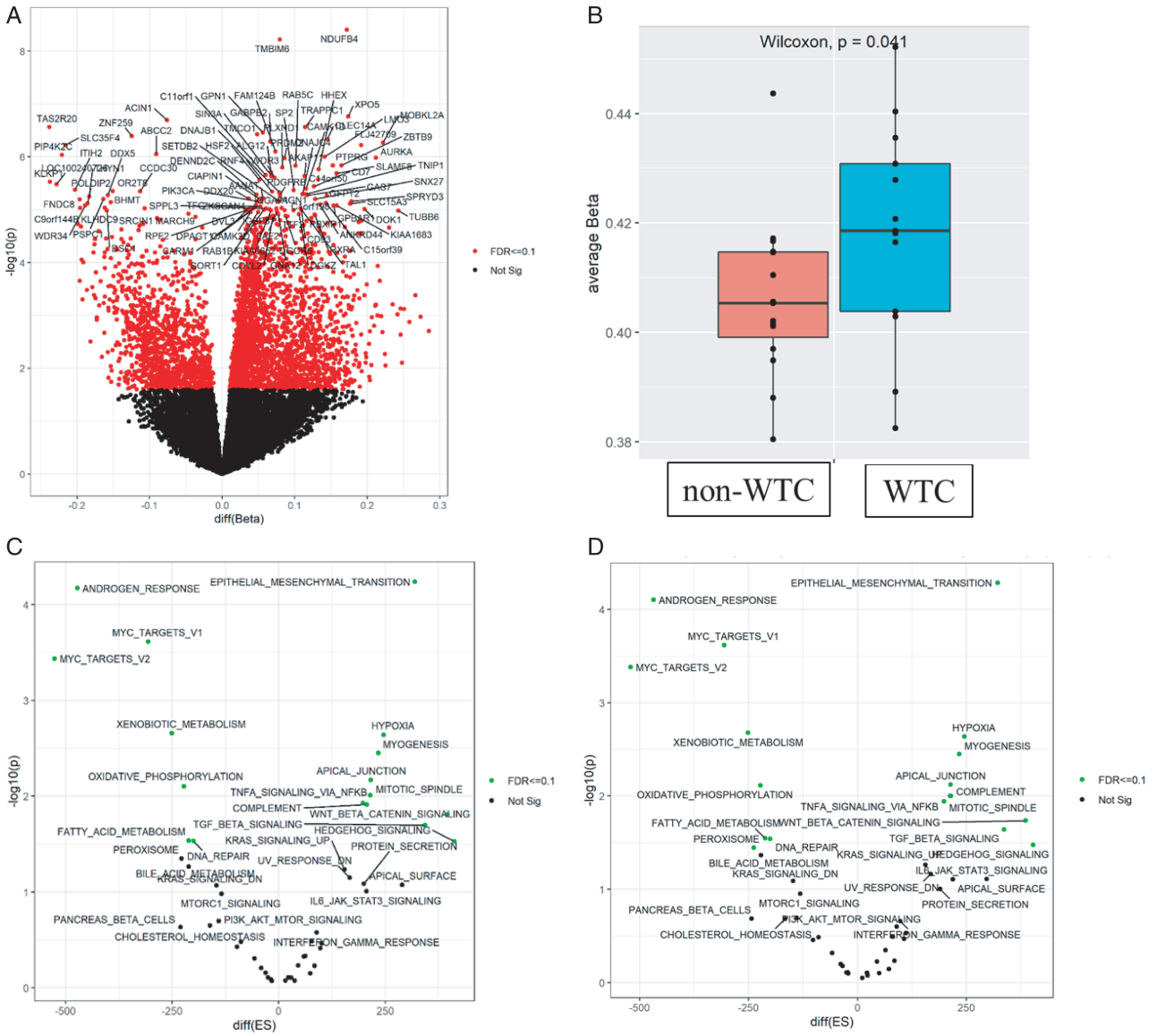


Figure 2. (A) Differentially methylated genes between WTC and non-WTC samples (unadjusted). The *x*-axis represents difference of Beta values between WTC and non-WTC samples, and a positive *x*-axis value indicates higher methylation in WTC samples. *y*-axis represents negative log10 of unadjusted *P*-values obtained from comparing WTC samples and non-WTC samples. The red color indicates adjusted *P*-values less than 0.1, which are considered significant. (B) Global methylation profiles of WTC vs non-WTC-exposed PCs. Rank-sum test between means of WTC samples and means of non-WTC samples on previous probes selected by most anti- correlation in TCGA (n = 14 042 probes). (C) Hallmark pathway enrichment results between WTC and non-WTC methylation profiles (unadjusted). The *x*-axis represents W-statistics from rank-sum tests between WTC and non-WTC samples; a positive *x*-axis value indicates hypermethylation in WTC samples. *y*-axis represents negative log10 of unadjusted *P*-values obtained from comparing WTC samples and non-WTC samples. *P*-values of pathways are calculated by rank-sum tests of in-vs-out-of-pathway (LIMMA) *t*-statistics from comparing expression between groups. The green color indicates adjusted *P*-values less than 0.1, which are considered significant. Genes are limited to hallmark pathways genes (n = 4336). ES, enrichment score. (D) Hallmark pathway enrichment results between WTC and non-WTC methylation profiles (adjusted for tumor purity). For color figure refer online version.

without WTC exposure included in the analysis were similar in terms of age at diagnosis, race/ethnicity and Gleason score.

Integrative analysis of DNA methylation

The comparison of methylation data of WTC and non-WTC-exposed samples showed significant differences in the methylation levels of 3586 genes (adjusted *P*-value < 0.1, see Methods for details). Among them, 2412 genes were hypermethylated and 1174 genes were hypomethylated in WTC compared to non-WTC samples (Figure 2A, Supplementary Figure 1, available at *Carcinogenesis* online). WTC samples had, on average, a mean methylation value of 0.418 compared to 0.406 in control samples; WTC samples were statistically significantly more hypermethylated (P = 0.04131) (Figure 2B).

Pathway analysis revealed 17 out of 50 pathways having statistically significantly differential methylation between WTC and non-WTC samples (adjusted *P*-value < 0.1). Of these, 10 pathways were significantly hypermethylated in WTC samples, while 7 were significantly hypomethylated (Figure 2C and Supplementary Table 1, available at *Carcinogenesis* online). The most significant hypermethylated pathways in WTC samples were the hypoxia, mitotic spindle,

TNFA signaling via NFKB, WNT signaling and TGF beta signaling pathways. The androgen response pathway was hypomethylated in WTC PC samples compared to non-WTC PC, as were the MYC targets pathways. These pathways enrichment results remained consistent even after adjustment by tumor purity (Figure 2D).

RNA-seq gene expression

Similar differential analysis between WTC and non-WTC groups at both gene and pathway level was performed using RNA-seq data. Forty-seven genes were differentially expressed between WTC-exposed and unexposed samples (Figure 3A, Supplementary Table 2, available at *Carcinogenesis* online). Of these, 24 were genes that were upregulated in WTC patients. Pathway enrichment analysis revealed that genes in the Myogenesis, Hypoxia, and TNFa signaling via NFkB pathways, among others, were expressed at lower levels in WTC samples compared to non-WTC samples. On the other hand, genes from multiple pathways including the androgen response pathway, G2M pathway, and MYC target pathway were more highly expressed in the WTC samples. Results were consistent before and after adjustment (Figure 3B & 3C).

Correlation of RNA-seq and methylation results

There was statistically significant inverse correlation between methylation data and RNA-seq gene expression data ($P < 0.0001$). For instance, the hypoxia pathway, which was shown to be hypermethylated in WTC samples compared to non-WTC samples, also showed lower gene expression in WTC samples. The androgen response pathway, which was

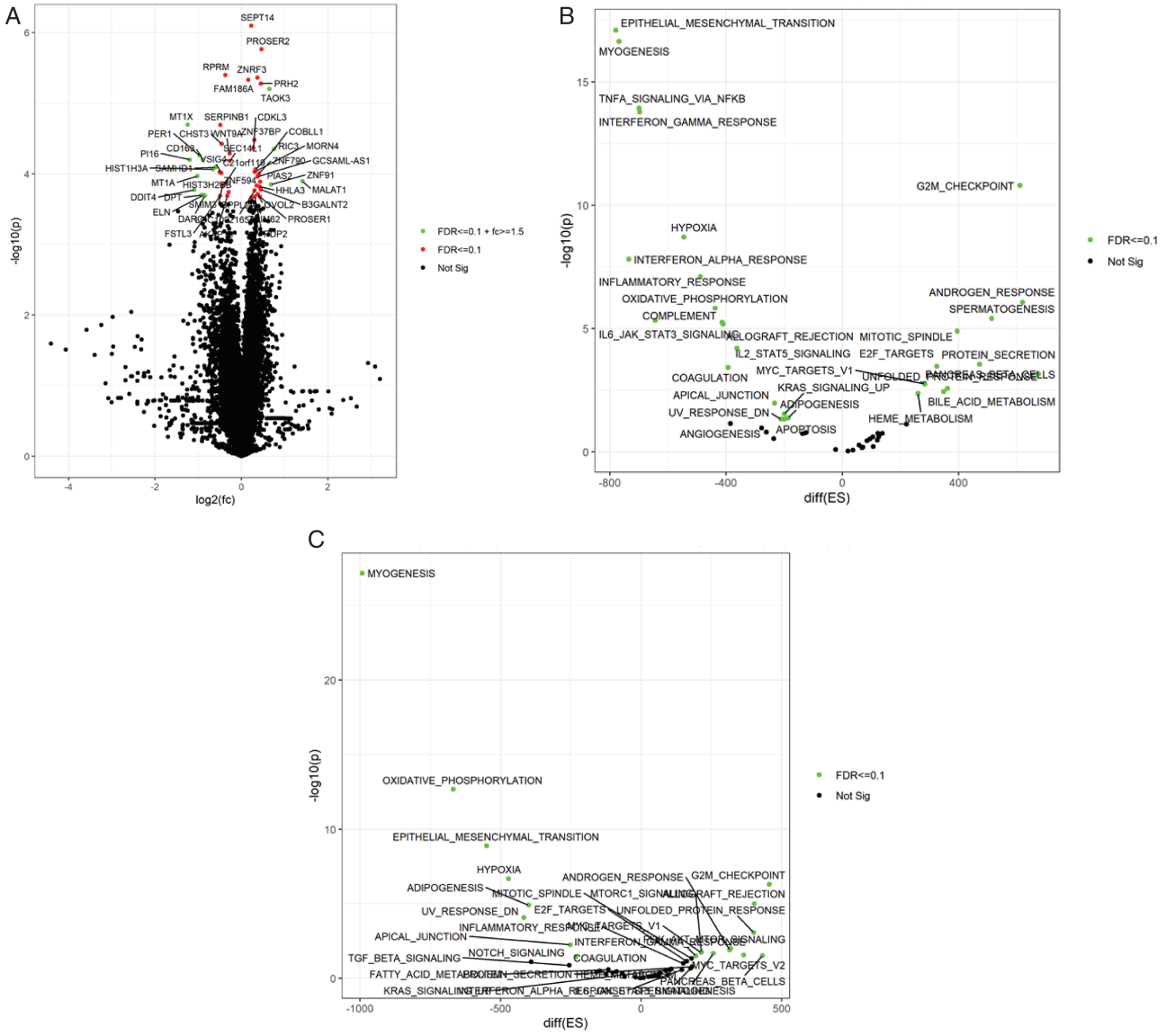


Figure 3. (A) Differentially expressed genes between WTC and non-WTC samples. The *x*-axis represents log2 of fold changes between WTC and non-WTC samples, and a positive *x*-axis value indicates higher expression in WTC samples. *y*-axis represents negative log10 of unadjusted *P*-values obtained from the comparison ofWTC and non-WTC samples. (B) RNA-seq gene expression analysis between WTC and non-WTC samples (unadjusted). Positive *x*-axis indicates higher gene expression in WTC patients. *P*-values of pathways are calculated by rank-sum tests of in-vs-out-of-pathway (LIMMA) *t*-statistics from comparing expression between groups. Genes are limited to hallmark pathways genes (n = 4336). (C) RNA-seq gene expression analysis between WTC and non-WTC samples (adjusted for tumor purity).

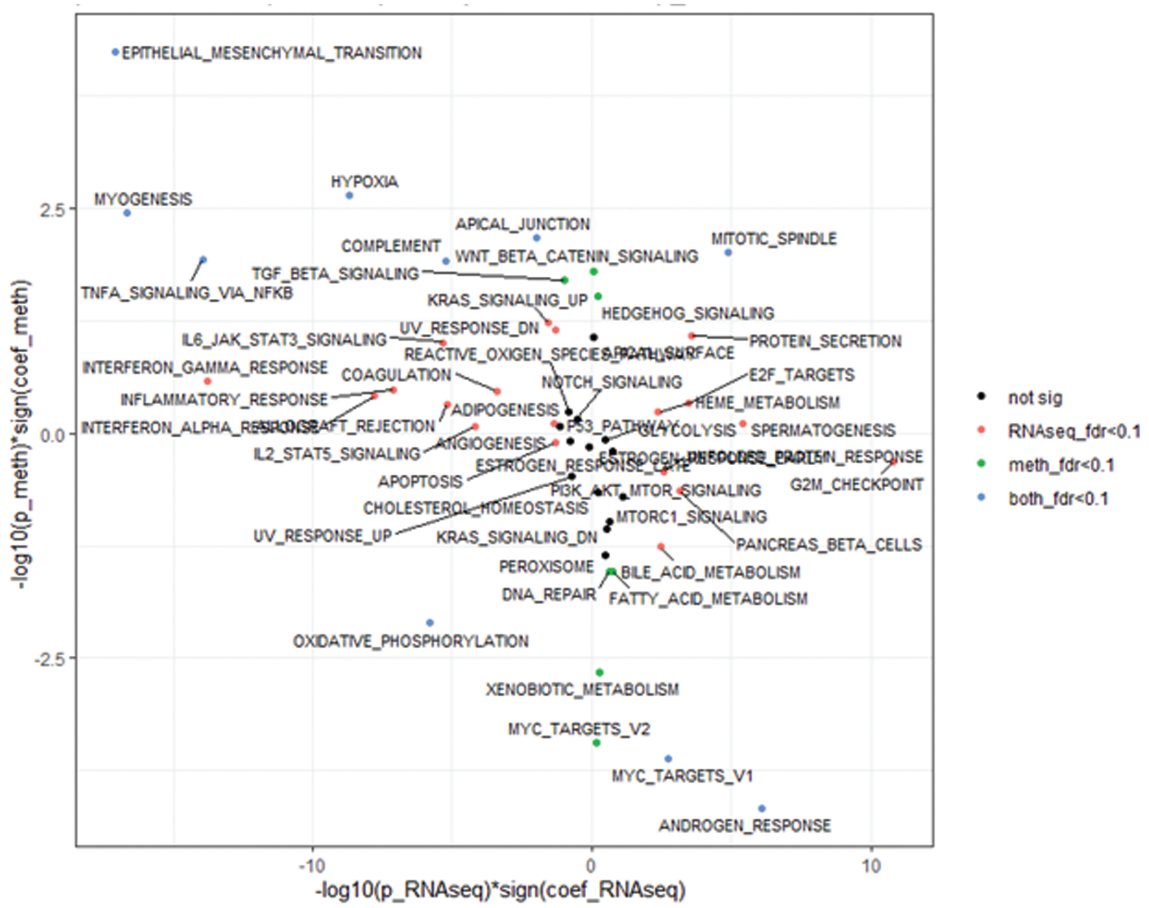


Figure 4. Correlation of RNA-seq and methylation pathway analysis. Positive *x*-axis is indicative of higher gene expression in WTC samples; positive *y*-axis shows hypermethylation in WTC samples. Results are adjusted for multiple testing. R = 5.13e–01; *P* = 1.42e–04.

shown to be hypomethylated in WTC PC patients, showed greater gene expression in WTC samples vs non-WTC samples (Figure 4).

Discussion

The results we present here contribute to the mounting evidence that WTC exposure resulted in epigenetic changes, which likely contributed towards carcinogenesis. Here, for the first time, we directly compared the DNA methylation profiles of WTC-exposed vs unexposed PC tumor tissue samples. Not only are there statistically significant differences in the methylation profiles of these two groups of PC patients, but there is increased global hypermethylation among WTC responders. Previously, Arslan et al. had reported similar findings when comparing the methylation profiles of healthy people exposed to WTC dust to unexposed controls, using DNA extracted from peripheral blood, whereby WTC-exposed subjects had increased global hypermethylation, and specifically increased methylation of key cancer genes (19). This work therefore builds on Arslan et al.'s by suggesting that persistent DNA methylation changes in cancer genes are present in both healthy and diseased WTC-exposed individuals, observable in blood and also in cancer tissues.

The observed hypomethylation in the androgen response pathway among WTC responders deserves attention. Androgen receptor (AR) signaling is critical to normal prostate homeostasis and PC progression (37,38). Inhibition of AR signaling, therefore, is a common first-line course of treatment of PC (38). However, response to androgen ablation therapy is variable, with 20–30% of PC cases becoming castration resistant through AR-dependent and -independent resistance mechanisms (38). The decreased androgen response pathway methylation, and increased gene expression, then, may be indicative of PC with greater capacity for proliferation (39). Since WTC and non-WTC groups were frequency matched on Gleason score, it seems unlikely that this finding is a product of confounding, although other criteria such as race, genomics, and treatment history are also important. Moreover, the DNA methylation profiles of WTC-exposed PC patients may have important implications for treatment response, although this requires further study.

We'd like to highlight, additionally, the upregulation of the G2M and MYC targets pathways. The G2M checkpoint is critically important to the cell cycle, dysregulation of which is associated with tumor cell proliferation (40). Similarly, MYC targets have functions related to proliferation, apoptosis, DNA damage repair and angiogenesis, among others (41). A consequence of this upregulation could be the faster growth of cancer cells within WTC PC tumors. The MYC pathway is in fact commonly over-activated in PC and is associated with PC progression and metastasis (42).

Interestingly, the EMT pathway was observed to be hypermethylated, with genes expressed at lower levels, among

WTC PC tumors compared to non-WTC tumors. As cancer cells grew faster in the WTC tumors, the stromal cell proportion would be correspondingly lower, resulting in the downregulation of the EMT pathway compared to non-WTC tumors. When WTC and non-WTC samples were compared, a higher frequency of PC cells were observed in WTC samples independently of the deconvolution methods used. Whether this is a biologically meaningful differences or an artifact of the sample collection process is not known. However, DNA was extracted in a blinded, uniform way by an expert pathologist. Gross macro-dissection on paraffin tissue sections was performed to maximize tumor content and targeted > 80% tumor purity, which for prostate oftentimes includes malignant stroma and epithelial cells but here focused on epithelial purity. That said, there is evidence for tumor-associated altered DNA methylation in adjacent normal, non-tumor cells. Previous research has shown that DNA methylation alterations of prostate tumors can be observed in peripheral tumor-associated prostate tissues, further decreasing the likelihood that tumor purity bias alone would explain our reported results (43). Additionally, we conducted a Gleason-to-Gleason comparison based on methylation data to further investigate the cancer cell number results, and saw that tumor epithelial cells were the only type of cells to significantly differ between WTC and non-WTC samples; levels of macrophages, B cells, CD4 + T cells, Natural Killer (NK) cells, CD8 + T cells, endothelial cells, eosinophils, cancer-associated fibroblasts, neutrophils and T regulatory cells (TREGS) were all comparable between the two groups ($P > 0.05$) when matched on Gleason score 6. For those samples rated as Gleason score 7 there was some difference in levels for B cells, CD4 + T cells, NK cells, CD8 + T cells, endothelial cells and cancer-associated fibroblasts. (Data not shown) We'd expect that imbalances in tumor purity would be dramatically reflected in the observed tumor immune response. To further support our findings, we undertook additional alternative methods of tumor purity adjustment for the hallmark pathway enrichment results. Specifically, we performed adjustment according to expression of epithelial cell markers EPCAM to assess and adjust for cancer cell abundance (Supplementary Figure 3A & B, available at *Carcinogenesis* online). When adjusting by EPCAM status, results were highly consistent with the results of the unadjusted analyses and of the initial method of tumor purity adjustment reported in Figures 2C, 2D & 3B, 3C. Since all the analyses point at an important new result, i.e. that WTC tumors contain more cancer cells on average, a possible sign of aggressiveness, adjusting for this important new finding could result in over-adjustment of the models. Nevertheless, we suggest that these findings require further exploration and validation in a larger sample size.

There are further limitations to the conclusions that can be drawn from this study, primarily due to the small sample size of this analysis; future studies using larger samples sizes are needed to validate the findings reported here. Because the WTC PC samples came from first responders, there is, additionally, the potential that the differences in methylation profiles and gene expression reported here could be due to other occupationally related exposures and not WTC dust exposure. Another limitation is that we lacked genetic information about these tumors, and therefore cannot comment on aneuploidy, which would likely influence results. Because we also did not explore the germline genetics of participants, we can also not exclude the possibility that WTC-exposed individuals harbored pre-existing genetic or epigenetic susceptibility to PC. Similarly, we are missing information on therapy and could not adjust for its possible influences on results. We hope to see these analyses repeated in future with larger studies that address these limitations.

Our results are strengthened, however, by the RNA-seq gene expression analysis performed using RNA from the same tumor section from which DNA was extracted for methylation profiling. The results from the RNA-seq gene expression analysis were statistically significantly correlated to the methylation results, increasing our confidence in these findings. Though complex, the most basic relationship between DNA methylation and gene expression is that genes with methylated promotors cannot be "turned on" and expressed due to steric hindrance caused by the methyl groups for the transcription machinery (44). Gene silencing through hypermethylation, while a normal part of development, also plays a major role in cancer (44). Although we are the first group to analyze DNA methylation and gene expression in WTC samples on the same tumor sample, the combination of small sample size and the degradation of the RNA over time likely limited the power to detect statistically significant results from gene expression analysis. The gene expression findings are confirmatory of our previous work, which revealed expression differences between WTC PCs and unexposed PC in immune system and inflammatory response genes (14). Here we confirmed decreased methylation among Th2 cells (Supplementary Figure 2, available at *Carcinogenesis* online), suggesting inflammatory dysregulation as a consequence of WTC exposure.

Epigenetic consequences of WTC exposures appear persistent and detectable many years after the original exposure. Multiple hypotheses could explain how inhalation of WTC dust affected epigenetic changes in distant organs such as the prostate. One attractive hypothesis is the maintenance of a chronic inflammatory response initiated in the lungs resulting in the systemic release of protumor cytokines that impact genetically susceptible organs such as the prostate. It is possible that WTC dust exposures initiated a local reaction in the lungs, and this precipitated distant epigenetic events with gene expression changes. Research has demonstrated that exposure to WTC dust induces increased inflammation and oxidative stress, and that these pathophysiological changes are associated with epigenetic modifications in the lungs of rodents (45). It is also possible that the fine particle nature of WTC dust allowed it to enter the bloodstream through the lungs and travel to distant organs, such as the prostate (8). Our previous research has shown that acute exposure to WTC dust through inhalation in rats is associated with changes in gene expression in prostate tissues, inducing a local inflammatory cascade and immune system suppression (14). The exact mechanism(s) remain unclear, yet it is becoming increasingly evident that epigenetic changes as a consequence of WTC dust exposure may play a role in PC development in WTC patients.

Conclusion

Our study demonstrates statistically significant differences in DNA methylation and gene expression in tumors of WTC PC patients compared to non-WTC PC. Pathway analysis revealed several candidate pathways through which WTC dust exposure may be associated with increased tumorigenesis

potential in prostate tissues. These data require replication and confirmation in expanded WTC cohorts of PC and other diseases types.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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Conflict of interest statement

No conflicts of interest.

Data availability

The data that support the findings of this study are available from the corresponding author, E.T, upon reasonable request.

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